Drug resistance detection and mutation patterns of multidrug resistant tuberculosis strains from children in Delhi

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ABSTRACT

A total of 312 sputum samples from pediatric patients presumptive of multidrug resistant tuberculosis were tested for the detection of drug resistance using the GenoTypeMTBDRplus assay. A total of 193 (61.8%) patients were smear positive and 119 (38.1%) were smear negative by Ziehl–Neelsen staining. Line probe assay (LPA) was performed for 208 samples/cultures (193 smear positive samples and 15 cultures from smear negative samples). Valid results were obtained from 198 tests. Of these, 125/198 (63.1%) were sensitive to both rifampicin (RIF) and isoniazid (INH). 73/198 (36.9%) were resistant to at least INH/RIF, out of which 49 (24.7%) were resistant to both INH and RIF (multidrug resistant). Children with tuberculosis are often infected by someone close to them, so strengthening of contact tracing in the program may help in early diagnosis to identify additional cases within the household. There is a need to evaluate newer diagnostic assays which have a high sensitivity in the case of smear negative samples, additional samples other than sputum among young children not able to expectorate, and also to fill the gap between estimated and reported cases under the program.

1. Introduction

Childhood tuberculosis (TB) is one of the major causes of childhood morbidity and mortality. An estimated 74,000 children die from TB each year and account for around half a million new cases annually worldwide. It is estimated that childhood TB constitutes 10–20% of all TB in high-burden countries [1].

In 2013, 63,919 pediatric TB cases were notified in India, under the Revised National Tuberculosis Control Program (RNTCP) [2]. Although child TB in India is estimated to be approximately 10% of the total adult incidence, only 6% of the total cases reported to the program are children. A large number of children with TB remain undiagnosed each year which makes it difficult to assess the actual magnitude of the childhood TB epidemic. Dodd and colleagues [3,4] modeled TB infection and estimated a prevalence of 50 million infected children. India is predicted to account for 27% of the total burden of pediatric TB in 22 countries with high disease burden. They interpreted that far more drug-resistant TB occurs in children than is diagnosed. According to their estimates, 850,000 children developed TB in 2014; 58,000 with isoniazid (INH) monoresistant TB, 25,000 with multidrug resistant (MDR) TB, and 1200 with extensively drug resistant (XDR) TB.

The challenges in diagnosing TB in children include the paucibacillary nature of the disease in many, difficulty to obtain a sample from young children, and low sensitivity of the commonly used smear microscopy technique. A molecular diagnostic test, GenotypeMTBDR plus Line probe assay (LPA; Hain Life Sciences, Nehran, Germany) was introduced in the RNTCP in 2011. The method is based on nucleic acid amplification directly from smear positive pulmonary specimens, permitting rapid detection of mutations in genes coding for resistance to rifampicin (RIF) and INH (Hain test). With LPA, turnaround time to diagnose MDR TB among smear positive pulmonary samples has decreased markedly [5,6].

Although the role of pediatric TB in the transmission of disease may be lower than that of adult patients, pediatric TB can be a reservoir which constitutes a significant number of future adult cases. Therefore, the epidemiology of the disease in children reflects the efficiency of the control programs and also enables bet-

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ter predictions of the resources required for management of children with TB/DR TB.

Since there is limited data on TB among children and its genetic determinants, the present study was conducted at the National Institute of Tuberculosis and Respiratory Diseases, National Reference Laboratory (NRL), to determine the proportion of MDR TB among South Delhi children presumptive of MDR and to detect associated mutations in *rpoB*, *katG*, and *inhA* genes using the GenoType MTBDRplus assay.

2. Materials and methods

2.1. Study setting

Delhi has an area of 1483 sq km, with a total population of 17 million and population density of 11,000/sq km. For the management of RNTCP, the state has been divided into 24 chest clinics. Under each chest clinic, there is one TB unit for half a million population having a designated microscopy center for every 0.1 million population. The Department of Microbiology, National Institute of Tuberculosis and Respiratory Diseases receives samples of presumptive MDRs from six South Delhi districts (population – 25 lakhs) for performing LPA under the program. A total of 312 samples from children ⩽15 years were received from six districts of South Delhi and outpatient departments during October 2011 to December 2013.

2.2. Patient demographic details and inclusion criterion

Sociodemographic characteristics of the study population were sourced from the referral for culture drug susceptibility testing (DST) forms and laboratory register. The data included age, sex, type of TB, and the presumptive MDR criterion of the patient.

The criteria for presumptive MDR-TB under the national program were: treatment failures among new TB cases, smear positive cases that remained smear positive after the 4th month of treatment with retreatment regimen, and pulmonary TB cases who were contacts of known MDR-TB cases (Criterion A). Criterion B included any smear positive follow up or smear positive retreatment case at diagnosis in addition to criterion A. Criterion C included smear negative retreatment cases and all HIV/TB co-infected cases at diagnosis in addition to Criteria A and B. Children who fulfilled the criteria for presumptive MDR-TB were screened in the peripheral DOT (Directly Observed Treatment) centers by medical officers and lab technicians, and referred to the lab for diagnosis.

2.3. Sample collection processing

Sputum samples (spot and morning) were collected from each patient in 50 mL wide-mouthed sterile falcon tubes. All specimens were screened for the presence of acid fast bacilli by Ziehl–Neelsen staining. The samples were processed by the N-acetyl-L cysteine–sodium hydroxide method [7]. Smear positive samples were subjected to LPA directly from processed samples. All smear negative samples were inoculated in MGIT 960 liquid culture tubes. Tubes which flashed positive were subjected to smear microscopy and to immune-chromatographic assay for detection of the mpt64 antigen to confirm the presence of the *Mycobacterium tuberculosis* complex. These cultures were further subjected to LPA.

2.4. LPA

All smear positive samples and smear negative culture positive isolates were subjected to the Genotype MTBDR V 2.0 plus assay as per the manufacturer's instructions. Each LPA strip had five control zones (conjugate, amplification, and a locus control each for *rpoB*, *katG*, and *inhA* genes). The test was considered as invalid in the case of a missing amplification band in a negative test result due to the presence of inhibitors or mistakes during amplification set up.

For RIF susceptibility determination, there were eight *rpoB* wild-type (WT1–WT8) and four mutant probes (MUT1 D516V, MUT2A H526Y, MUT2B H526D, and MUT3 S531L). For INH susceptibility determination, *katG* WT with two mutant probes (MUT1 S315T1 and MUT2 S315T2), and two *inhA* WT with four mutant probes (MUT1 C15T, MUT2 A16G, MUT3A T8C, MUT3B T8A) were present. Either missing of the WT band or the presence of a mutant band was taken as an indication of a resistant strain. The presence of all WT probes with no signal from the mutant probe was considered as sensitive. The presence of all wild type probes along with the presence of one or more mutant bands was considered as hetero resistant.

3. Results

3.1. Acid fast bacilli microscopy and culture

A total of 312 children who fulfilled the criteria of presumptive MDR and whose sample was sent for DST were analyzed for the study. The distribution patterns regarding age groups (years) and male to female sex-ratios were: 0–5 $n = 2$ (1/1); 6–10 $n = 43$ (11/32 or 0.34); 11–15 $n = 267$ (58/209). The mean age of patients was 13 years with 85.5% (267/312) of children in the age group of 11–15 years. Overall, there were more females than males with a male to female sex-ratio of 70/242 or 0.28 (Table 1). The proportion of cases between 0–15 years was 4–6% (312/5663) of the total presumptive MDR cases received for culture and DST from different districts (Table 1). All patients were retreatment cases: Category (Cat) I failure ($n = 13$); Cat II failure ($n = 4$); retreatment cases before starting Cat II treatment ($n = 91$); any follow up positive during Cat I or Cat II treatment ($n = 89$), smear negative retreatment cases ($n = 101$); contact of MDR patients ($n = 1$); and no information regarding criterion ($n = 13$).

Of the total patients, 193 (61.8%) were smear positive and 119 (38.1%) were smear negative. Smear positivity was higher among the 11–15 year age group (179/267; 67%) followed by those aged 6–10 years (24/43; 55.8%). Of 119 smear negative samples, 15 (12.6%; 5 boys and 10 girls) were culture positive for *M. tuberculosis* complex. Of these there was one patient each of 9 and 12 years of age, two patients each of 10, 11 and 13 years, three patients of 15 years and four patients of 14 years of age.

3.2. Drug susceptibility and mutation patterns

Samples/cultures of 208 children were subjected to LPA (193 directly from samples and 15 from culture). Valid results were obtained from 198 tests. Ten invalid samples were either scanty ($n = 4$) or 1+($n = 6$). Of the 198, 125 (63.1%) were sensitive to both RIF and INH; 49 (24.7%) were found to be resistant to RIF and INH, 6 (3.0%) as mono-RIF resistant, and 18 (9.1%) as mono-INH resistant. In all, 27.8% (55/198) children were resistant to RIF (Table 1).

The mutation pattern for RIF and INH resistance using GenoTypeMTBDRplus is presented in Table 2. Among 55 RIF resistant strains, the commonest mutation was at codon S531L of the *rpo*B gene (41/55; 74.5%) followed by H526Y (3/55; 5.5%) and D516V (2/55; 3.6%). In 10 (18.2%) RIF resistant strains, resistance was determined by absence of one or more wild type probes with no gain in mutant probe. Hetero-resistance to RIF was found in seven samples (12.7%), with S531L being the most common mutation.

Table 1
Demographic details and resistance patterns obtained from different districts of South Delhi.

Districts	Samples of children ⩽15/total samples of MDR suspect received (%)	Male (%)	Female (%)	Sm+ (%)	Sm− (%)	Valid LPA	IR sensitive (%)	IR resistant (%)	Mono R resistant (%)	Mono I resistant (%)
NITRD	91/1636 (5.56)	23 (25.3)	68 (74.7)	53 (58.2)	38 (41.8)	55	37 (67.3)	9 (16.4)	3 (5.4)	6 (10.9)
BJ	30/538 (5.57)	8 (26.7)	22 (73.3)	16 (53.3)	14 (46.7)	15	12 (80)	3 (20.00)	0	0
DFIT	55/865 (6.35)	12 (21.8)	43 (78.2)	38 (69.1)	17 (30.9)	39	25 (64.1)	12 (30.8)	0	2 (5.1)
MN	57/1146 (4.97)	13 (22.8)	44 (77.2)	28 (49.1)	29 (50.9)	31	14 (45.2)	13 (41.9)	0	4 (12.9)
RTRM	27/636 (4.24)	3 (11.1)	24 (88.9)	18 (66.7)	9 (33.3)	19	16 (84.2)	1 (5.3)	0	2 (10.5)
MT	10/199 (5.02)	2 (20.0)	8 (80.0)	9 (90.0)	1 (10.0)	8	4 (50.0)	3 (37.5)	1 (12.5)	0
NA	42/643 (6.53)	9 (21.4)	33 (78.6)	31 (73.8)	11 (26.2)	31	17 (54.8)	8 (25.8)	2 (6.5)	4 (12.9)
Total	312/5663 (5.5)	70/312 (22.4)	242/312 (77.6)	193/312 (61.9)	119/312 (38.1)	198	125/198 (63.1)	49/198 (24.7)	6 (3.0)	18 (9.1)

BJ = Bijwasan; DFIT = Damien foundation India Trust; I = isoniazid; LPA = line probe assay; MN = Malviya Nagar; MT = Moti Nagar; NA = non area (outpatient department (OPD) patients); NITRD = National Institute of TB and Respiratory Diseases; RTRM = Rao Tula Ram Memorial; Sm+ = smear positive; Sm− = smear negative; R = rifampicin.

Table 2
Pattern of gene mutations in resistant *Mycobacterium tuberculosis* strains using the Genotype MTBDR*plus* assay.

Gene	WT probes; +/−	MUT probes +/−	Codon	Gene region/mutation	No. of samples (RIF^R n = 55; INH^R n = 67)
rpoB	WT8−	MUT3+	S531L	530–533	35
		M1+, M3+	D516V, S531L	506–509, 530–533	1
		No mutant probe			1
	All WT+	MUT3+	S531L	530–533	4
		MUT1+	D516V	506–509	1
		MUT2A	H526Y	526–529	1
		MUT2B+, MUT3+	H526D, S531L	526–529, 530–533	1
	WT3−, WT4−	No mutant probe		513–517, 517–519	4
	WT7−, WT8−	MUT2A	H526Y	526–529, 530–533	1
	WT7−	MUT2A	H526Y	526–529	1
		No mutant probe			2
	WT2−	No mutant probe		510–513	1
	WT2−, WT3−	No mutant probe		510–513, 513–517	2
Kat G	WT−	MUT1+	S315T1	315	58
	WT+	MUT1+	S315T1	315	6
	WT−	No mutation	S315T1	315	2
InhA	WT1−	MUT1+	C15T	−15	1

MUT = mutant; +/− = present/absent; R = resistant; WT = wild type.

Among 67 INH resistant strains, high level resistance corresponding to a mutation in codon 315 of the *Kat G* gene occurred in 98.5% (66/67) of samples, whereas low level resistance (C15T mutation) in the −15-promoter region was present in a single strain. Hetero-resistance with both WT and mutant pattern to INH was found in 9% (6/67) strains (Table 2).

4. Discussion

The majority of children, 267/ 312 (85.5%) were in the age group of 11–15 years. Some recent studies have shown that adolescents are a vulnerable group with a higher chance of developing the disease compared to young children [8,9]. In the present study, the majority of children in the higher age group may be due to selection bias, as children below 6 years are not able to expectorate the sputum. Obtaining specimens like gastric lavage aspirate, induced sputum, and bronchoalveolar lavage is challenging, which highlights the fact that presumptive TB and DR-TB among younger children and infants may be underdiagnosed.

Most of the children had adult type sputum positive or even MDR pulmonary TB with a high prevalence of disease in girls (77.5%, *p* < 0.001). It is confirmed that adolescent girls have a greater tendency to develop adult type sputum smear positive TB than boys [10]. In both sexes, the highest risk occurs during the peak of adolescent growth spurts. The development of TB is also often associated with menarche [11] and to the hormonal and metabolic perturbations of puberty. A recent study from Iran has similar findings and showed that prevalence of TB increased by increasing age and the population of female patients also rose [12]. Similar findings of another study showed that the smear positive pediatric TB was found more often in adolescent girls than boys of the same age [13].

Children constituted around 5% of the total presumptive MDRs referred for DST and were Cat I/Cat II failures/any follow-up smear positives/smear negative retreatment cases. Only a single child was enrolled as contact of MDR. Current World Health Organization guidelines advise that all children <5 years of age who are in close contact with a sputum smear positive index patient should be actively traced, screened for TB, and provided preventive chemotherapy after active TB has been excluded [14]. Contact tracing needs strengthening in most high burden settings, including India. Presently, the coverage is less than satisfactory as internal evaluations conducted between 2012 and 2014 noted that 35–70% of children aged 6 years did not receive chemoprophylaxis, highlighting the need to prioritize this activity [15]. Because children with TB have often been infected by someone close to them, contact tracing and drug susceptibility testing of adults with TB

can provide crucial data to inform treatment of children with non-microbiologically confirmed TB and of apparently healthy children with latent TB infection.

38% of cases were smear negative and culture positivity among these was low (12.6%). The LPA can be set up at the IRL/NRL level, as it requires sophisticated laboratory equipment, quality control measures, trained human resources, and backup of solid/liquid culture to manage specimens that are smear negative. Although the use of LPA reduces the turnaround time in smear positive samples, smear negative and extrapulmonary samples must first be cultured prior to genotypic analysis. There is a need to evaluate newer diagnostic assays which have a high sensitivity in the case of smear negative samples for this vulnerable group. The present study included only sputum samples. Since the rates of extrapulmonary, especially meningeal, TB are high in children, we need to emphasize equally on extrapulmonary samples [16]. Recently, a pilot study using upfront Xpert MTB/RIF testing for diagnosis of TB in a pediatric population in respiratory and extrapulmonary specimens was conducted in India. It showed a twofold increase in TB case detection over smear microscopy and detection of significant numbers of RIF-resistant TB cases [17].

RIF resistance was detected in 27.8% (55/198) samples with valid LPA results. The resistance detected was high as compared to an earlier study by Singhal et al. [6] which mainly included an adult population (RIF resistance 459/2038; 22.5%).

74.5% (41/55) of the strains harbored a mutation in codon S531L similar to other studies from Delhi (59–72%) [6,13]. Among 67 INH resistant strains, all except one had a mutation at codon 315 corresponding to high level resistance. In contrast, *InhA* mutations were found in 13.4–17% and *katG* mutation occurred in around 88% of INH resistant strains in the previous studies from Delhi [6,18].

A total of 18 cases (9.1%) were monoresistant to INH. The figures are almost similar to mono INH resistance found among adults (unpublished data from the laboratory). Notably, these patients would not be diagnosed by newer diagnostic tests which only test for rifampin resistance. Since INH resistance increases the likelihood of poor outcomes including treatment failure, population based studies are required to determine the true incidence of INH resistance among different epidemiological areas [19].

As compared to adults, children with drug resistant TB usually have transmitted resistance, and MDR cases among children represent recent infection with drug resistant strains. DST patterns among children provide important information regarding current transmission patterns in the community and implementation of control programs. The relation between strain genotype and clinical manifestation of disease is poorly documented in children. Our preliminary findings demonstrated that SIT1/Beijing (28/90 or 31.1%), followed by SIT26/CAS1-Delhi (27/90 or 30%) were predominant among children from Delhi. The same SITs were predominant, although with different percentages in adult XDR patients, which reflects the considerable transmission of these genotype families in this setting [20,21]. Epidemiological surveys from South Africa demonstrated that pediatric MDR TB tripled over the last 15 years (2.3–6.7%) and the main mode of acquisition was primary transmission of a drug resistant strain [22]. If newly diagnosed TB cases in children are put to first line therapy based on smear examination, some of the primary resistant cases among children may be missed/not detected. This in turn may expose large numbers of children to inadequate TB treatment, leading to risk of treatment failure, development of further resistance, and increased mortality. Recently, all pediatric cases presumptive of TB have been prioritized for testing with molecular diagnostics (CBNAAT/LPA) as per standards of TB care in India. However, identification of children with TB/MDR-TB has gaps, and further training and sensitization of both private and public pediatricians and health care workers is required to improve access to diagnostic and treatment services.

Consideration of additional samples like gastric aspirate and induced sputum in the case of children not able to expectorate can further help in confirming a correct diagnosis among this group.

Since the study was conducted in a routine program setting it is likely to reflect the reality in the field. The limitations of the present study were the small number of samples received and since 104/312 (33.3%) of children were smear negative culture negative, the data underestimate the true burden of TB in this group. Second line DST data was not available for all patients and hence not projected in the manuscript. More studies with complete resistance profiles to all first and second line drugs among children are required, given the fact of high fluoroquinolone resistance present in India.

The present study , although limited by the small sample size, is however concerning, and additional studies are needed to more accurately define the prevalence of such resistant strains in both pulmonary and extrapulmonary samples among this vulnerable population.

With the introduction of new and emerging technologies, universal access to DST of all TB patients, to allow for individualized, DST guided drug regimens becomes more feasible. Active tracing and screening of household contacts at high risk would allow children with the disease to receive early diagnosis, thus reducing complications. Active case finding and new strategies to improve case detection among smear negative patients and increased access to care are required for better management of TB in children.

Conflict of interest

None declared.

Acknowledgements

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